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UNIVERSITY OF ALBERTA

TISSUE GLUCOSE UPTAKE IN THE INSULIN RESISTANT JCR:LA-cp RAT

by

© LEANNE M. WENZEL

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

EXPERIMENTAL SURGERY
DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

SPRING 1994

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled, *Tissue Glucose Uptake in the Insulin Resistant JCR:LA-cp Rat,* submitted by Leanne M. Wenzel in partial fulfillment of the requirements for the degree of Master of Science in Experimental Surgery.

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April 15, 1994

To the memory of my father and mother,
James and Doreen Foxley

ABSTRACT

The JCR:LA-corpulent rat carries an autosomal recessive gene that induces massive obesity in homozygous (cp/cp) animals, while heterozygous (+/cp) or homozygous (+/+) normal rats are lean. The obesity is accompanied by an extreme insulin resistance, impaired glucose tolerance, and hyperinsulinemia, all of which are more marked in the male rats. There is also an accompanying hypertriglyceridemia due to hypersecretion of very low density lipoproteins (VLDL). We have determined the relative uptake of glucose in the liver and peripheral tissues using the non-metabolizable tracer, 2-[^{3}H]-deoxy-D-glucose (2DG), under both basal and hyperinsulinemic conditions. Delivery of 2DG via portal vein injection under basal conditions showed no significant differences in 2DG uptake in the liver, diaphragm, heart, or epidydimal fat in the male and female cp/cp animals compared to the lean males. Glucose uptake in the perirenal fat in the cp/cp males and in the subcutaneous fat in both the male and female corpulent animals was decreased in comparison to the +/+ rats under basal conditions. Similar results were obtained for experiments using jugular vein delivery. Under hyperinsulinemic conditions, the +/+ males showed a sharp increase in glucose uptake in the skeletal muscle. No increases were seen in the liver, heart, or fat. The cp/cp males, in contrast, showed no change in glucose uptake under hyperinsulinemic conditions. The cp/cp females showed increased uptake in the skeletal muscle that was less than that shown by the +/+ rats. These results confirm the existence of a profound insulin resistance of the cp/cp male rat which lies primarily in muscle. The cp/cp females are less severely impaired under conditions of hyperinsulinemia than the cp/cp

males. Both corpulent genotypes are able to compensate for their insulin resistance under basal insulin conditions and to normalize glucose uptake in peripheral tissues. Age-related changes in glucose uptake were observed mainly as reductions in uptake by adipose tissue and likely reflect the low rates of growth and fat deposition in the six-month-old animals. Results of Southern blot analysis of the insulin receptor gene indicate that the insulin resistance observed in the JCR:LA-cp rat is not due to large rearrangements, insertions, or deletions in the insulin receptor gene. Further studies must be completed before any conclusions about the origin of the insulin resistance in the JCR:LA-cp rat can be made.

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CHAPTER 1

INTRODUCTION

The obesity/insulin resistant/hyperlipidemic syndrome has been recognized as a major risk factor for the development of cardiovascular disease and, in particular, atherosclerosis. In his Banting lecture in 1988, Reaven described the disorder characterized by android obesity, hypertriglyceridemia, peripheral insulin resistance, and hypertension and designated it as "Syndrome X".[1] The syndrome is associated with atherosclerosis, a condition which underlies the cardiovascular disease. Thus it has become very important to understand the underlying metabolic defects associated with this syndrome. Both the mechanisms leading to the abnormal metabolism and obesity and the origin of the vascular and myocardial disease remain obscure. Until recently, the study of this syndrome had been slow, partly due to an absence of acceptable animal models. Interest in the fa/fa Zucker rat and, more recently, in strains of rats incorporating the cp gene has led to new approaches for examining this disease process.

The JCR:LA-corpulent rat is one of the strains incorporating the cp gene originally isolated by Koletsky.[2-4] Animals homozygous for the cp gene (cp/cp) are obese and display a range of metabolic abnormalities which include a pronounced hyperlipidemia and insulin resistance.[5,6] Animals which are heterozygous (cp/+) and homozygous normal (+/+) are phenotypically lean and appear to be metabolically normal. While the cp gene has been incorporated into five other strains of rat, only the JCR:LA-cp strain spontaneously develops both atherosclerosis and ischemic myocardial lesions.[7-9] The lesions are confined to the cp/cp male animals, with the lean

rats and cp/cp females being unaffected, at least up until middle age. In the cp/cp rats, the presence of hypertension is not related to the cardiovascular disease, with the normotensive JCR:LA-cp being markedly affected and the hypertensive SHR/N-cp being unaffected.[7] The corpulent rats have a marked VLDL hyperlipidemia which is far more pronounced in the females than in the male animals.[10,11] The hyperlipidemia is due to hepatic hypersecretion of VLDL, but not of LDL or HDL.[12,13] The male cp/cp rats have a greater hyperinsulinemia and impaired glucose tolerance than the cp/cp females.[14] Recent studies suggest that the obese rats have a normal basal glucose turnover, but have a profound insulin insensitivity in the peripheral tissues.[15] These rats provide an efficient, relatively economical model for the study of the common syndrome in man characterized by obesity, mild type II diabetes, and hypertriglyceridemia that leads to a high risk of cardiovascular disease.

The origins of both the obesity and the hypersecretion of VLDL remain obscure, but are clearly related to the insulin resistance and abnormal glucose metabolism. A first requirement to understanding the syndrome is to identify the origin of the excessive triglyceride production and fat accumulation. The cp/cp rat appears to divert the majority of metabolic glucose, derived from a carbohydrate rich diet, to triglyceride. This is then secreted into the plasma as VLDL and transported to the peripheral tissues. The core defect appears to lie in the insulin/glucose metabolism of both the peripheral tissues and the liver.

In order to gain a better understanding of the nature of the insulin resistance in the JCR:LA-cp rat, it is important to examine possible abnormalities within the insulin receptor itself. The insulin receptor is regulated by many factors, and alterations in the insulin receptor may lead to

the abnormalities in insulin action observed in insulin resistant states.[16] Possible mechanisms of insulin resistance may include defects at the prereceptor, receptor, or postreceptor levels.[17] Prereceptor defects would include changes in the structure or function of insulin and could result from antibodies against insulin or a mutation in the insulin gene itself.[17] Receptor defects could result in a decrease in receptor number, receptor affinity or a decrease in tyrosine kinase activity.[16] Postreceptor defects would include abnormalities in glucose transporters, glucokinase activity, or other intracellular pathways of insulin action.[17] In persons with NIDDM, abnormalities in the action of insulin have been demonstrated at both the receptor and postreceptor levels.[17,18] The recent cloning of the human insulin receptor cDNA[19,20] has allowed the investigation of possible genetic alterations in the insulin receptor gene which might play a role in syndromes of insulin resistance. The purpose of this study was:

(1) to identify the primary site of insulin resistance in the JCR:LA-cp rat; and

(2) to investigate the possibility that the insulin resistance observed in the JCR:LA-corpulent rat is due to a genetic alteration of receptor function and, in particular, gross changes in the insulin receptor gene.

CHAPTER 2

LITERATURE REVIEW

THE INSULIN RESISTANCE SYNDROME

Insulin resistance can be defined as a condition in which the body's tissues fail to respond normally to the action of insulin.[21] The association of non-insulin-dependent diabetes mellitus (NIDDM), obesity, hypertension, atherosclerotic cardiovascular disease, and dyslipidemia with insulin resistance and hyperinsulinemia has been well established over the past decade.[21] A great deal of evidence now exists to indicate that these metabolic abnormalities are part of a syndrome. Reaven[1] originally designated this disease process as "Syndrome X"; however, it has also been labelled, the "Insulin Resistance Syndrome."[21] According to Defronzo,[21] insulin resistance due to obesity or some other primary genetic defect results in a compensatory increase in the secretion of insulin by the β cells of the pancreas. As long as the β cells are functioning in a normal manner, there will be a compensatory increase in the secretion of insulin which precisely offsets any defects in insulin action.[21] The profound hyperinsulinemia leads to a downregulation of insulin receptor numbers, resulting in a more severe state of insulin resistance.[22] If the β cells for some reason cannot maintain the secretion of insulin at the levels necessary to offset the severe insulin resistance, a frankly diabetic state will inevitably result.[21] This theory appears to be the most generally accepted theory for the development of NIDDM in humans.

It has been shown in some individuals that the persistent stimulation of the β cell to hypersecrete insulin can lead to a progressive deterioration in β cell function. Some studies suggest that glucose toxicity due to chronic hyperglycemia may lead to an acquired defect in β cell secretion of insulin resulting in insulin levels which are insufficient to offset the insulin resistance.[22] Chronic hyperglycemia might lead to a downregulation of the glucose transport system resulting in a defect in insulin action at the level of insulin dependent tissues and an impairment in insulin secretion at the level of the β cell.[23] Insulin resistance is not only a result of obesity but can also lead to its development.[21] Insulin resistance in skeletal muscle leads to a resultant hyperinsulinemia which enhances the hepatic synthesis of lipid. This excess lipid is delivered to adipose tissue and is stored as triglyceride. The hyperinsulinemia also enhances the uptake of glucose by adipocytes which results in enhanced triglyceride synthesis. Obesity is the end result.[21]

An alternative argument would state that the hyperinsulinemia precedes the development of the insulin resistance. McGarry[24] has hypothesized the existence of an unidentified inherited factor causing the pancreas to hypersecrete insulin resulting in hepatic lipogenesis. Excessive production of VLDL and delivery to muscle and fat depots would lead to triglyceride deposition in both tissues. The accumulation of fat in the muscle would interfere with storage and oxidation of glucose resulting in muscle insulin resistance. Episodic postprandial hyperglycemia would further stimulate β cell function setting up a "vicious cycle" resulting in glucose intolerance. Failure of β cell function by unknown mechanisms would inevitably result in NIDDM.[24] Much evidence exists to support the argument that increased insulin secretion by the β cells of the pancreas precedes the development of an insulin resistant state. First, patients with insulinoma

showed degrees of insulin resistance which were directly proportional to their degree of hyperinsulinemia.[24] Second, Cusin et al.[25] demonstrated that hyperinsulinemia imposed on normal rats leads to most abnormalities observed in animal models of obesity linked with hyperinsulinemia, including hyperphagia, increased glucose utilization in adipose tissue, increased de novo lipogenesis in both adipose tissue and liver, increased weight gain, and muscle insulin resistance. Third, Zarjevski et al.[26] showed that muscle insulin resistance may not be the primary etiological factor in the fa/fa Zucker rat. Finally, overexpression of the human insulin gene in transgenic mice resulted in insulin resistance following the initial hyperinsulinemia.[24]

In both obese humans and animals the primary cause of insulin hypersecretion remains to be elucidated. In the case of obese humans, a primary insulin resistance in the peripheral tissues with a secondary hyperinsulinemia leading to the development of a severe state of insulin resistance cannot be excluded.[27] However, this is likely not the case in obese animal models, since hyperinsulinemia is one of the earliest abnormalities detected.[27] There are several possible causes of hyperinsulinemia in obese states. First, hypothalamic abnormalities have been described in genetically obese animals.[27] Second, lesions of the ventromedial hypothalamic (VMH) area result in a hyperinsulinemic state within minutes. It has been suggested that the CNS may modulate the endocrine portion of the pancreas via the vagus nerve. Hyperactivity of the vagus nerve follows lesions of the VMH and causes a reduction in the release of somatostatin and an increase in the release of both insulin and glucagon from the pancreas.[27] Third, a hyperactivity of the enteroinsular axis, and in particular an increased secretion of the insulin secretagogue gastric inhibitory peptide (GIP) has been suggested

as a possible cause of hyperinsulinemia in obese humans.[27] Fourth, a dysfunction in the endocrine pancreas itself could be the primary source of hyperinsulinemia in certain types of obesity.[27] Finally, because of the strong association between hyperphagia and hyperinsulinemia, it is possible that the hypersecretion of insulin could simply be the result of hyperphagia. However, evidence exists which demonstrates that hyperinsulinemia and obesity may develop independent of hyperphagia.[27] Clearly, more studies are needed in this area before any conclusions can be made regarding the origins of hyperinsulinemia in obese humans and animal models.

The Role of Insulin Resistance and Hyperinsulinemia in the Development of Coronary Artery Disease

Prospective epidemiological studies have demonstrated that both fasting and postprandial hyperinsulinemia are risk factors for the development of coronary artery disease (CAD) in individuals who are non-diabetic, and that this relationship is independent of obesity, hypertriglyceridemia, hypercholesteremia, decreased physical activity, hypertension, and smoking.[21] Clinical studies have also shown positive correlations between insulin and fibrinogen and plasminogen activator inhibitor.[28] There is now a great deal of evidence which suggests that insulin may be an atherogenic hormone.[21] The advanced atherosclerotic lesion or fibrous plaque is covered by a fibrous cap consisting of dense connective tissue which surrounds flattened smooth muscle cells and macrophages. This fibrous layer covers an assortment of smooth muscle cells, lymphocytes, and macrophages containing variable

amounts of lipid.[29] Experimental evidence suggests that plasma insulin levels can affect all of these constituents.[21] Proliferation of cultured smooth muscle cells is dramatically increased by incubation in the presence of insulin. The addition of insulin both in vivo and in vitro increased the activity of low density lipoprotein receptors and increased cholesterol and triglyceride synthesis in arterial smooth muscle cells, fibroblasts, and mononuclear cells.[21] Insulin has also been shown to promote the synthesis of collagen, a very important component of the connective tissue matrix of the advanced atherosclerotic lesion. Insulin also stimulates the production of growth factors which cause cell proliferation and thus contribute to the development of atheromatous plaques.[21] Not only does insulin play a direct role in the formation of such plaques, but hyperinsulinemia inhibits the reabsorption of these lesions once they have been formed.[21] Although it has been demonstrated that insulin plays an important part in the formation of atherosclerotic lesions, the existence of hyperinsulinemia suggests the existence of an insulin resistant state, and thus it is important to consider the insulin resistance syndrome as a whole when examining the pathogenesis of CAD.[21] An insulin resistant state causes a shift in metabolism from glucose to lipid which might also contribute to the development of atherosclerosis. CAD may be the result of a combination of hyperinsulinemia and insulin resistance. The alteration in cellular metabolism caused by the insulin resistant state coupled with the direct vascular affects of insulin may favour the development of accelerated atherosclerosis.[21]

THE BIOSYNTHESIS OF INSULIN

The human insulin gene is a 1.5 kbp sequence located on the short arm of chromosome 11. The gene includes three exons. Exons 2 and 3 code for preproinsulin, a long chain peptide (MW 11,500), which contains the signal peptide (24 amino acids), the insulin B chain (30 amino acids), and the insulin A chain (21 amino acids).[17]

Preproinsulin is produced by ribosomes on the rough endoplasmic reticulum of the pancreatic β cells and is cleaved by microsomal enzymes to proinsulin (MW 9000) which is transported to the Golgi apparatus in microvesicles and packaged into clathrin-coated secretory granules.[30] Proinsulin typically contains 86 amino acids and includes the A and B chains of insulin as well as a connecting segment (C peptide) consisting of approximately 35 amino acids.[30] Proinsulin can vary in length from 78 amino acids to 86 amino acids depending on the length of the C peptide region.[31] Proinsulin which escapes cleavage is secreted into the bloodstream with insulin and C peptide. Proinsulin is biologically much less active than insulin (7-8% the biological activity of insulin), however, it does cross react with most of the anti-insulin sera used in routine insulin immunoassays.[31] Proinsulin is degraded by the kidneys and is not removed by the liver, which means it has a half life 3-4 times that of insulin. Because proinsulin is able to accumulate in the blood, it accounts for approximately 12-20% of the circulating insulin in the basal state in humans.[31] Conversion of proinsulin to insulin and C peptide is completed in the secretory granules by proteolytic cleavage involving enzymes with trypsin and carboxypeptidase B activity. C

peptide is released from pancreatic β cells in equimolar amounts along with insulin and has no apparent biological activity.[30]

INSULIN STRUCTURE

Insulin consists of two polypeptide chains consisting of 51 amino acids. The A chain contains 21 amino acids and the B chain contains 30 amino acids. The chains are connected by two interchain disulfide bridges and a third intrachain disulfide bridge which links positions 6 and 11 of the A chain.[31] The two chain structure has been preserved throughout evolution. However, some major differences in sequence have been observed between species. The majority of mammalian insulin molecules have similar sequences, excluding the guinea pig and the coypu, in which insulin sequences differ by 35-40%. Throughout evolution however, the primary structure of the insulin molecule has remained relatively stable.[32]

INSULIN SECRETION

The human pancreas secretes approximately 40-50 units of insulin per day. Secretion of insulin requires energy and involves the microtubule-microfilament system in the β cells of the pancreas.[31] It is believed that mature secretory granules attach to microtubules which contract in the presence of high levels of intracellular calcium, resulting in the ejection of insulin containing granules from the β cells via exocytosis.[30]

Basal insulin secretion is the amount of insulin secreted under fasting conditions. Plasma glucose levels below 80-100 mg/dl do not stimulate the release of insulin. A maximal response to insulin occurs at glucose levels between 300 and 500 mg/dl.[31] Insulin secretion in response to exogenous stimuli results in a biphasic response. The first phase of insulin secretion (acute secretion) results in the release of stored insulin and reaches a first peak within a few minutes, followed by a reduction in secretion despite the continued presence of the stimulus.[32] The second phase includes secretion of stored insulin but when this phase is prolonged newly synthesized insulin is required. The second phase occurs more slowly but reaches a higher level of secretion.[32]

A number of mediators responsible for stimulating insulin release have been identified and include direct stimulants which stimulate insulin release directly as well as amplifiers which potentiate the β cell response to glucose. Direct stimulants include glucose, mannose and the amino acid leucine. Vagal stimulation as well as pharmacologic agents such as the sulfonylureas have also been shown to directly stimulate insulin release.[30] Amplifiers of glucose induced insulin release include enteric hormones such as gastric inhibitory polypeptide, cholecystokinin, secretin, and gastrin. Other amplifiers include neural amplifiers (β-adrenergic stimulation) and the amino acid arginine.[30] Glucose has been shown to be the most potent stimulator of insulin secretion. However, it is not well understood how the presence of glucose stimulates insulin release from the β cells of the pancreas. One hypothesis suggests that glucose may bind to a receptor located on pancreatic β cells, resulting in insulin secretion. However, no such receptor has yet been identified.[31] Because agents like 2-deoxy-D-glucose, which inhibit the

metabolism of glucose, interfere with the release of insulin, it has been suggested that it is the metabolism of glucose itself which is necessary for stimulating insulin secretion.[30] In fact, there is some experimental evidence which suggests that β cell glucokinase acts as the glucose sensor and is thus responsible for regulating the secretion of insulin.[33]

THE INSULIN RECEPTOR

The insulin receptor is composed of two α subunits (apparent MW 120,000-130,000) and two β subunits (apparent MW 90,000) which combine to form a disulfide-linked heterodimer (MW 350,000-400,000).[16,19,31,32,34,35] The α subunit is completely extracellular and appears to be the insulin binding subunit. In contrast, the β subunit is a transmembrane glycoprotein which appears to be an insulin sensitive protein kinase involved in signal transduction.[16,31] Both the α and β subunits of the insulin receptor contain complex carbohydrate side chains whose functional significance has not yet been determined. Removal of both sialic acid and galactose, however, results in decreased insulin binding.[16]

It appears that the insulin receptor is synthesized from a single chain glycosylated pro-receptor with a molecular weight of approximately 190,000.[16,19,31,32,34,35] The pro-receptor is initially synthesized in the rough endoplasmic reticulum, and is glycosylated and cleaved to yield mature α and β subunits within the Golgi before being transported to the plasma membrane.[16,19] The human insulin receptor gene is located on chromosome 19.[31] Biosynthesis of the insulin receptor requires approximately 1.5 to 3

hours.[16] The half-life of the mature insulin receptor is approximately 7 to 12 hours.[16,34] Receptors are internalized and are either degraded or recycled back to the plasma membrane. A single receptor may be recycled several times before it is actually degraded.[16] Insulin can regulate receptor numbers presumably by accelerating receptor degradation, a process referred to as down-regulation.[36] Other factors which can influence the number of insulin receptors include exercise and diet, as well as hormones including growth hormone, and the glucocorticoids.[36]

Insulin receptors can be found on most mammalian cells in concentrations of up to 20,000 per cell.[16] The location of the insulin receptor is not restricted to tissues typically regarded as classical target tissues of insulin and this likely reflects the complexity of its actions.[16] The role insulin plays in the regulation of glucose metabolism is well known. In the liver, insulin inhibits gluconeogenesis and glycogenolysis and promotes glycogenesis, while in muscle and adipose tissue insulin promotes the uptake, storage, and use of glucose.[36] Insulin, however, is also involved in the growth and replication of cells, tissue repair and regeneration as well as organogenesis and differentiation in fetal development.[31] The basic structure and binding properties of the insulin receptor are virtually identical in all cells and species which have been studied.[16] Evolutionarily, the structure of the insulin receptor is even more highly conserved than that of insulin itself.[31]

INSULIN ACTION

Insulin action is initiated once the hormone binds to the insulin receptor on the surface of the target cell. When insulin binds to its receptor this results in a conformational change in the receptor, a rapid aggregation of receptors, internalization of the hormone-receptor complex, an increase in ion flux, and a translocation of insulin sensitive glucose transporters from an intracellular pool to the plasma membrane.[16] This is followed by a change in the activity of various enzymes and changes in the synthesis of certain macromolecules.[16] Insulin's actions can occur within seconds or minutes (transport, protein phosporylation, enzyme activation and inhibition, and RNA synthesis) or after several hours (protein and DNA synthesis and cell growth).[31] The initial event in insulin action may be the activation of the β subunit tyrosine-specific protein kinase. Recent studies have demonstrated that insulin binds to the α subunit of the insulin receptor, which causes the autophosphorylation of the β subunit and activation of the β subunit tyrosine-protein kinase.[32] The β subunit acts as a protein kinase in transferring the gamma phosphate of ATP to a tyrosine residue in the β subunit.[31] An ATP binding site exists on the β subunit, which suggests that this subunit is the kinase of the receptor.[16] Insulin increases the V_{max} of this enzyme.[16] There are several lines of evidence which suggest that activation of the tyrosine-specific protein kinase of the β subunit is the initial step in insulin action. First, insulin, in physiological concentrations, stimulates the phosphorylation and tyrosine-specific kinase of its receptor within 60 seconds. This process is rapidly reversible.[32] Second, the kinetics of the phosphorylation and dephosphorylation reactions of the insulin receptor are

consistent with the time course of insulin action.[16] Third, site directed mutagenesis of the kinase domain has demonstrated that the tyrosine kinase activity of the β subunit is essential for insulin's metabolic and growth promoting actions as well as for receptor internalization.[37] Fourth, the microinjection of monoclonal antibodies which inhibit tyrosine kinase activity also inhibit insulin action.[38] Finally, tyrosine protein kinase activity is very rare in mammalian cells, and appears to be associated with proteins which are essential in the regulation of cellular growth and metabolism.[16] Other proteins containing tyrosine kinase activity include EGF, PDGF, IGF-I, and gene products of several of the transforming RNA viruses.[16]

The essential role of tyrosine kinase in insulin action appears to be generally accepted. However, it is not known exactly how activation of this kinase results in signal transduction and subsequent biological events.[31] However, it is clear that the well known mechanisms of signal transduction including cyclic nucleotides and ion channels are not involved in insulin action.[39] Studies on protein phosphorylation have demonstrated that insulin stimulates both protein phosphorylation and dephosphorylation. This observation has led to some confusion regarding the mechanisms of insulin action.[39] Following stimulation of receptor tyrosine kinase activity, an array of protein kinases which phosphorylate specific serines and threonines within regulatory proteins are activated.[40] There is evidence for the existence of an insulin activated serine protein kinase which can phosphorylate and activate the protein phosphatase responsible for the dephosphorylation of glycogen synthase and phosphorylase kinase.[40] Thus, the activation of serine and threonine protein kinases may be essential for all of the intracellular protein phosphorylations which result following occupation of the insulin receptor.[40]

It has been hypothesized that these kinases are part of a kinase cascade. However, the event or events which might lead to an initiation of these cascades following activation of the insulin receptor kinase remain to be elucidated.[40]

The search for a second messenger of insulin action has been underway since the early 1970s.[39] Initially, the search for a substance capable of mediating some of the actions of insulin resulted in the isolation of two substances which were released from the plasma membranes of hepatocytes in response to insulin.[39] These substances were capable of inhibiting adenylate cyclase and stimulating cAMP phosphodiesterase. Thus, it was thought that these substances might play a role in reducing intracellular levels of cAMP, and would therefore have a significant influence on the actions of insulin in the liver.[39] A low molecular weight substance with an inositol glycan structure which mimics some of insulin's actions on important metabolic enzymes has been identified.[39] The substance is produced by an insulin sensitive hydrolysis of a glycosyl-phosphatidylinositol (glycosyl-PI) in the plasma membrane, catalyzed by a specific phospholipase C (PLC).[39] The hydrolysis reaction also produces a diacylglycerol which selectively regulates one or more of the protein kinases C.[39] Two theories have been proposed for how activation of the glycosyl-PI specific PLC is coupled to the activity of the insulin receptor.[39] First, activation of glycosyl-PI PLC by the receptor might result from a tyrosine kinase induced cascade causing changes in the state of phosphorylation of the enzyme or perhaps a regulating factor.[39] Second, autophosphorylation of the β subunit tyrosine residues might signal the initiation of a membrane coupling event involving non-covalent interactions with a regulatory factor.[39] In either case, the regulatory factor for

the proposed coupling event might be a GTP binding protein (G protein) which could activate the PLC.[39]

The role of diacylglycerol and the involvement of calcium and protein kinase C in insulin action are not completely understood. Studies have demonstrated that phorbol esters (tumor promoting activators of protein kinase C, which can substitute for diacylglycerol) can mimic insulin's actions on glucose transport and oxidation, lipogenesis, pyruvate dehydrogenase, mitogenesis, and the phosphorylation of certain proteins.[39] However, phorbol esters inhibit insulin stimulated lipogenesis and and interfere with insulin's actions on glycogen synthesis and glycogenolysis.[39] It has been suggested that insulin may lead to a selective activation of kinase C by structurally distinct diacylglycerols.[39] This selective activation of protein kinase C might help to explain the above observations in which protein kinase C activators such as phorbol esters mimic some, but not all, of the effects of insulin.[41] The inositol glycan looks very promising as a second messenger of insulin action. However, further studies are clearly required before the role of these compounds is fully understood. The development of inhibitors and perhaps studies of site directed mutagenesis might be helpful in determining the functional role of the glycosyl-PI PLC reaction in the pleiotropic actions of insulin.[39]

Evidence exists which suggests that insulin itself may carry out some of its actions within the cell interior.[42] It has been well established that the hormone-receptor complexes are internalized by target cells and the receptors are recycled back to the cell surface.[42] Inhibition of insulin internalization in adipocytes inhibits insulin stimulated glucose transport.[42] It has been demonstrated that insulin plays a direct role in regulating functions involved

in RNA processing and transport in isolated rat liver nuclei and nuclear membranes.[42] The direct interaction of insulin with the nucleus might help to explain how insulin regulates RNA metabolism and DNA synthesis.[42]

From the above summary on insulin action it is clear that the intracelluar pathways involved in the actions of this hormone are extremely complex and not fully understood. Further research will be required to elucidate the mechanisms involved so that we may gain a better understanding of the diverse actions of this hormone.

GLUCOSE TRANSPORTERS

Glucose is a major energy source in mammals and the metabolism of this substrate provides a constant supply of ATP in both anaerobic and aerobic conditions. The storage of glucose as glycogen in the liver allows for the regulation of blood glucose levels, while glycogen stores in muscle function as energy storage sites for exercising muscle.[43] Glucose taken up by adipocytes can be converted into triglycerides which can be stored and broken down to fatty acids and glycerol when required.[43] Because of its important role, defects in the uptake or metabolism of glucose can have detrimental effects on cellular metabolism which can result in severe morbidity or mortality as seen in diabetes mellitus.[43] Clearly, it is necessary for us to have a better understanding of the proteins responsible for transporting glucose across the plasma membrane. There are many transporter proteins which together serve to maintain glucose homeostasis by synchronizing both the absorption and utilization of glucose by all cells in the body.[44] The

Na^+/glucose cotransporters and the facilitative glucose transporters are the two main classes of glucose transporters which have been identified in mammals. The Na^+/glucose cotransporters are responsible for the active uptake of glucose against its concentration gradient, while the facilitative glucose transporters mediate the passive transport of glucose down its concentration gradient.[43]

FACILITATIVE GLUCOSE TRANSPORTERS

The facilitative glucose transporters are a family of structurally related proteins which transport glucose across the plasma membrane via facilitated diffusion. Glucose transport utilizing these proteins does not require ATP and is saturable, stereoselective and bidirectional.[43] The direction of transport depends on the relative concentration of glucose on either side of the membrane. Because glucose is quickly phosphorylated by either hexokinase or glucokinase, there is typically a net inward flux of glucose in most tissues.[43]

The cDNAs which encode the five functional isoforms of the facilitative glucose transporters have been isolated and characterized from human tissues.[43] These isoforms have been labelled GLUT which is the gene symbol for the facilitative glucose transporters. Each isoform is identified with a number which is based on the chronological order of publication of the cDNA sequence.[43] Recently, a pseudogene (GLUT 6) has been isolated from human tissues. GLUT1, GLUT2, GLUT3, and GLUT4 have been isolated from other species including mice and rats. The GLUT5 cDNA has been

found to cross-hybridize with mouse DNA which suggests that this particular isoform will be discovered in other species.[43] The human GLUT6 probe will not cross-hybridize with mouse DNA, however, which suggests that this pseudogene may be species specific.[43]

GLUT1 Isoform:

The GLUT1 glucose transporter isoform was the first to be cloned and sequenced. The cDNA of this isoform was used to screen cDNA libraries from a number of different cells which eventually resulted in the discovery of the GLUT2, GLUT3, and GLUT4 isoforms.[44] The amino acid sequence of the GLUT1 isoform is highly conserved evolutionarily. There is an approximately 98% identity between GLUT1 sequences in human and rat, and a 97% identity between the sequences of human and mouse, rabbit or pig.[43] Based on the amino acid sequence, it has been hypothesized that the GLUT1 transporter (MW 54,000) has twelve transmembrane spanning domains with the N- and C-terminus facing towards the cytoplasm.[44] The remaining facilitative glucose transporters have similar structures to the GLUT1 transporter. The major differences between transporters have been observed in the cytoplasmic domains and between transmembrane domains 6 and 7.[44]

Although GLUT1 is found in high levels in fetal tissues, brain, and placenta, it can be detected in most tissues. GLUT1 is distributed equally between the plasma membrane and an intracellular vesicle population. GLUT1, like GLUT4 is translocated to the plasma membrane, however, quantitatively the intracellular pool of GLUT1 is not sufficient to make any significant contribution to insulin stimulated glucose transport.[43] The GLUT1 isoform has a high affinity for glucose and is virtually saturated at

physiological glucose concentrations.[44] This glucose transporter may be responsible for non-insulin-stimulated glucose uptake. The GLUT1 transporter may play a role in the transport of glucose across blood-tissue barriers.[43]

GLUT2 Isoform:

The GLUT2 isoform is found in high levels in the β cells of the pancreas and in the liver. Lower levels of this transporter are found in the small intestine and the kidney.[43] The GLUT2 transporter has a high capacity and low affinity for glucose and is not saturated by physiological blood glucose concentrations.[44] Thus glucose transport is not the rate limiting step for glucose utilization in tissues expressing this particular transporter.

The GLUT2 isoform is the predominant glucose transporter found in the liver and is responsible for both the uptake and release of glucose.[43] Studies have demonstrated that the expression of GLUT2 is insulin independent and thus glucose transport in hepatocytes is not affected by insulin.[45] It has also been shown that GLUT2 mRNA and protein levels are not dramatically affected by modifying metabolic conditions.[43] Jongsik et al.[45] have demonstrated that rat hepatocytes possess an intracellular storage pool for GLUT2 but have no insulin responsive glucose transporter translocation mechanism.

The GLUT2 isoform is also found in high levels within the β cells of the islets of Langerhans. In the β cells an increase in the intracellular metabolism of glucose results in the stimulation of insulin secretion.[44] Recent studies have shown reduced GLUT2 mRNA and protein levels in islets from rats with both acquired and genetic forms of NIDDM.[43] Thus, it has been suggested that the GLUT2 isoform is an important component of the β cell

glucose sensor, and that a decrease in the expression of this transporter could partially account for the β cell failure observed in diabetes mellitus.[43]

GLUT3 Isoform:

GLUT3 mRNA has been isolated in all tissues examined thus far in adult humans, and thus together with GLUT1 may be responsible for non-insulin-stimulated glucose transport.[43] This isoform is found in high levels in the brain, kidney and placenta. In monkeys, rats, rabbits, and mice GLUT3 mRNA levels are very high in the brain but are extremely low or undetectable in other tissues.[43] It appears that in the brain both GLUT1 and GLUT3 are responsible for the uptake and disposal of glucose. GLUT1 is responsible for the transport of glucose across the blood-brain barrier, while GLUT3, which has a higher affinity for glucose than GLUT1, transports glucose directly into neuronal cells.[43]

GLUT4 Isoform:

The GLUT4 isoform is the insulin-responsive glucose transporter and is found predominantly in muscle (skeletal, smooth, and cardiac) and adipose tissue (brown and white).[43] Insulin does not alter the affinity (K_m) of the transporter for glucose but instead increases the transport velocity (V_{max}).[43] Under basal conditions the GLUT4 isoform is mostly confined to an intracellular vesicle population and thus does not contribute appreciably to glucose transport.[43] Insulin stimulation results in the fusion of these vesicles with the plasma membrane resulting in an increase in the number of glucose transporters associated with the plasma membrane.[43] The presence of insulin

stimulates glucose transport in peripheral tissues by causing the recruitment or translocation of GLUT4 transporters from an intracellular pool to the plasma membrane.[43,44] This translocation is energy dependent and requires ATP.[43]

GLUT5 Isoform:

The GLUT5 isoform is found in high levels in the jejunal region of the small intestine, but can also be detected in human kidney, skeletal muscle, and adipose tissue.[43] This transporter may be responsible for the uptake of dietary glucose from the lumen of the small intestine, or it could play a role in the active uptake of glucose.[43] Further studies are needed to resolve the functions of this transporter isoform.

THE PHYSIOLOGICAL BASIS OF INSULIN RESISTANCE

Insulin resistance is defined as a condition in which the body's tissues fail to respond normally to the action of insulin.[21] Because NIDDM and obesity are the most common diseases associated with insulin resistance, and because population and familial studies have demonstrated that heredity plays a role in both diseases, it has become very important to investigate the molecular defects responsible for initiating both disease processes.[17] A better understanding of the actions of insulin has allowed the mechanisms of insulin resistance to be divided into three distinct groups, including prereceptor, receptor, and postreceptor insulin resistance.[17] Prereceptor insulin resistance could result from defects preventing insulin from coming into contact with the

insulin receptor and might include changes in the structure or function of insulin resulting from the presence of anti-insulin antibodies or from a mutation in the insulin gene.[17] Receptor insulin resistance would result from a decrease in the number of receptors on the cell surface, a decreased affinity of insulin binding, or a defect in receptor tyrosine kinase activity.[17] Postreceptor insulin resistance might result from defects in glucose transport or other intracellular pathways of insulin action.[17]

Alterations in the structure or function of insulin are most typically the result of anti-insulin antibodies, but mutations of the insulin gene have also been observed.[17] Mutations in the insulin gene which result in an absence of C-peptide cleavage result in familial hyperproinsulinemia, while mutations responsible for the production of abnormal insulin result in insulinopathies.[17] Prereceptor defects do not appear to be associated with the insulin resistant states observed in obesity and NIDDM.

Mutations in the insulin receptor gene which decrease the number of insulin receptors on the cell surface have been linked to three different types of molecular defects and include mutations that impair receptor biosynthesis (transcription of the insulin receptor gene), mutations that impair the transport of receptors to the cell surface (posttranslational processing of insulin receptor), and mutations that accelerate receptor degradation.[38] Mutations which impair receptor function have been identified and include mutations that decrease the affinity of the receptor for insulin and mutations that impair tyrosine kinase activity.[38] Most of the mutations described above are associated with rare syndromes of severe insulin resistance including type A insulin resistance, leprechaunism, and Rabson-Mendenhall syndrome. However, a mutation in the tyrosine kinase domain of the insulin receptor has

been described in a form of NIDDM associated with a very severe insulin resistance.[46] Mutations of the insulin receptor gene which have been detected thus far have been within the structural gene itself and the majority of these mutations have been single nucleotide substitutions.[36] Mutations in the gene promoter or other non-coding domains have not been described.[36] A decrease in the number of insulin receptors has been observed in various tissues of nondiabetic obese persons and lean or obese persons with NIDDM.[36] No molecular defect in the biosynthesis of the insulin receptor has been described in these patients thus far.[47] However, it has been demonstrated that the tyrosine kinase activity of insulin receptors derived from fat, skeletal muscle, and liver is impaired in patients with NIDDM, and that weight loss in obese patients with NIDDM improves this defect.[36] In this case the defect in receptor function appears to be the result of an abnormal metabolic state and not a primary cause of the insulin resistance.[36] Because insulin levels appear to correlate inversely with the number of insulin receptors in these disease states, it might be assumed that this down regulation of receptor number is responsible for the insulin resistant state.[47] Because of the severity of the insulin resistance observed in obese patients and patients with NIDDM and because modest decreases in receptor number cannot account for this severity, it has been hypothesized that defects distal to the insulin-receptor complexes must exist.[48]

Because of the existence of "spare receptors," even if insulin binding is decreased as much as 60%, meaning that there is a 60% decrease in receptor numbers, there should still be an adequate number of receptors remaining in the muscle or adipose cells to allow maximal stimulation of glucose metabolism.[48] Thus, a decrease in receptor number may not be

sufficient to cause the severe insulin resistance observed in obesity and NIDDM. It is for this reason that postreceptor defects are suspected of contributing to the insulin resistance observed in these disease states. Although postreceptor defects could include any number of proteins in the intracellular pathways of insulin action, studies of the insulin-dependent glucose transporter GLUT4 appear to be the most common. Human studies of obesity and NIDDM have demonstrated profound decreases in GLUT4 mRNA and protein levels in adipose cells.[49] Furthermore, these decreases in transporter levels involved plasma membrane and intracellular compartments.[50] Because it is skeletal muscle and not adipose tissue which is the major contributor to insulin mediated glucose uptake, studies of GLUT4 expression in skeletal muscle have proved to be very interesting.[51] The changes observed in the expression of GLUT4 in adipose cells are not seen in studies of skeletal muscle.[49] Studies in both obese humans and patients with NIDDM have demonstrated that GLUT4 content in several muscles was similar in these subjects compared to controls.[50,51] It has therefore been hypothesized that insulin resistance in skeletal muscle might be due to impaired function or translocation and not to a depletion of transporters as seen in adipose tissue.[50] Studies are being carried out to determine if impaired translocation, fusion with the membrane, or activation of GLUT4 transporters in skeletal muscle might be responsible for insulin resistant glucose transport.[49] Results of studies on GLUT4 expression in hyperinsulinemic, insulin resistant rodent models resemble those in humans. Studies of obese Zucker rats showed no changes in GLUT1 or GLUT4 mRNA or protein levels in skeletal muscle.[51] Studies of obese, diabetic db/db mice showed no changes in GLUT4 expression in comparison to leans in both skeletal muscle

and adipose tissue.[52] Mutations in the glucokinase gene are suspected of contributing to the development of NIDDM and studies are being carried out to demonstrate the existence of alterations in this gene.[17] Studies of insulin resistant genetically obese fa/fa rats demonstrate an impaired activation of protein kinase C in the heart and liver of these animals.[53] Downregulation of protein kinase C in lean rats by pretreatment with phorbol myristate acetate (PMA) led to insulin resistance and unresponsiveness to PMA.[53] The results suggest that a portion of the insulin resistance observed in the fa/fa rats might result from an altered modulation of insulin action by protein kinase C.[53] Decreased activity of the enzyme phosphatidate phosphohydrolase-2 (PAP-2) was observed in hearts and livers of obese, insulin resistant, male and female JCR:LA-cp rats.[54] PAP-2 is involved in the formation of diacylglycerol and thus decreased activity of this enzyme could result in decreased diacylglycerol formation and activation of protein kinase C.[54] A decrease in protein kinase C activation might interfere in signal transduction and could thus contribute to the insulin resistance observed in these animals.[54] Clearly, a better understanding of the intracellular pathways of insulin action is required before more questions about the origins of insulin resistance in obesity and NIDDM will be answered.

THE CONTRIBUTION OF CORTISOL AND FATTY ACIDS TO INSULIN RESISTANCE

It has been demonstrated that both cortisol and fatty acids can decrease tissue sensitivity to insulin.[55] Glucocorticoids can cause insulin

resistance directly by decreasing the number of glucose transporters or indirectly by increasing the levels of glucagon and free fatty acid (FFA).[36] In contrast to insulin, cortisol promotes the breakdown of proteins and stimulates lipolyis via the actions of the catecholamines.[56] Thus cortisol leads to an increase in both FFA and gluconeogenic precursor levels. Cortisol also increases the activity of phosphatidate phosphohydrolase and thus contributes to increased triacylglycerol production.[56] Evidence suggests that an increased sensitivity to cortisol action may be an important factor in the development of both android obesity and insulin insensitivity.[57]

The increase in fat mass observed in obese individuals with and without NIDDM leads to an increased rate of lipolysis which inevitably results in an increase in the FFA concentration in the plasma.[23] Increased FFA concentration results in an increase in the uptake of FFA and stimulates the oxidation of lipid. The increase in FFA oxidation leads to an accumulation of acetyl CoA in the cell. Acetyl CoA is a powerful inhibitor of pyruvate dehydrogenase.[23] Because the NADH/NAD ratio is increased, the TCA cycle is slowed resulting in an accumulation of citrate. Citrate inhibits the enzyme phosphofructokinase resulting in an inhibition of the early steps of gycolysis.[23] Levels of glucose-6-phosphate increase and inhibit hexokinase activity resulting in a decrease in the transport of glucose into the cell. Thus, it is clear that in the muscle, increased lipid oxidation inhibits insulin mediated glucose disposal.[23] In the liver, the increase in FFA oxidation stimulates gluconeogenesis and thus increases hepatic glucose production.[23] Increased FFA levels can also activate the enzyme phosphatidate phosphohydrolase and thus also contribute to increased triacylglycerol synthesis.[55] Increased hepatic triacylglycerol synthesis results in a greater

secretion of VLDL from the liver.[55] In addition to the effect of FFA on glucose transport in muscle, it has also been shown that FFA can prevent insulin from binding to its hepatic insulin receptor directly resulting in hepatic insulin resistance.[57] From the above summary it is clear that both cortisol and FFA play important roles in the development and maintenance of the insulin resistance syndrome.

Obese Rodent Models

The Origins of the Corpulent and Fatty Rat Strains:

The two major autosomal recessive mutations resulting in obesity and a wide range of metabolic defects in the rat are the corpulent (cp) and the fatty (fa) mutations. Both mutations arose spontaneously in outbred stocks and have been backcrossed onto inbred strains.[4] The cp mutation appeared following a number of generations of inbreeding from a cross between an SHR/N female and a normotensive Sprague Dawley rat.[4] The SHR rat was produced by crossing an outbred Wistar male rat with spontaneous hypertension to a female with slightly elevated blood pressure.[4] Breeding pairs were distributed to the National Institute of Health (NIH) following the thirteenth generation of inbreeding.[4] The *N* denotes the NIH and is used to distinguish this strain from other SHR lines.[4] The LA/N-cp and the SHR/N-cp were developed by backcrossing the cp mutation onto the LA/N and SHR/N strains. The SHHF/Mcc-cp, derived from the SHR/N-cp, was developed and described by Dr. Sylvia McCune, Ohio State University, Columbus, and has developed a number of different characteristics than the SHR/N-cp and is not

considered to be a true congenic strain.[4] The cp gene has also been backcrossed onto the WKY/N strain which was initially developed from an outbred Wistar Kyoto stock.[4] At the fifth generation backcross to the LA/N, the LA/N-cp strain was distributed to Dr. James C. Russell, University of Alberta, and has been maintained as an outbred colony.[4] Because of its unique characteristics, this strain has been designated as JCR:LA-cp to distinguish it from the true congenic strain (LA/N-cp). The fa mutation developed spontaneously in an outbred colony of rats at the Laboratory of Comparative Pathology, Stow, Massachusetts.[4] The fa gene has been backcrossed onto a number of strains; however, only the Zucker-fatty strain will be discussed.

Metabolic Characteristics of the Corpulent and Fatty Rat Strains:

The SHR/N-cp is obese, hyperinsulinemic, insulin resistant, hyperlipidemic, glucose intolerant, and mildly hypertensive and exhibits both glycosuria and proteinuria.[58,59,60] The SHHF/Mcc-cp, like the SHR/N-cp, is obese. However, rats of this strain are hypertensive and develop a spontaneous cardiomyopathy resulting in heart failure in 75% of the males from as early as 11 months of age, and in 30% of the females at 24 months of age or greater.[61] LA/N-cp rats are obese, normoglycemic, hyperinsulinemic, insulin resistant, and hypertriglyceridemic.[60] This strain does not develop any overt cardiovascular disease. The characteristics of the JCR:LA-cp rats have already been described above. Like the LA/N-cp and the JCR:LA-cp, the Zucker-fatty rat is obese, normoglycemic, hyperphagic, hyperinsulinemic, insulin resistant, and hypertriglyceridemic.[58,62]

Although similarities do exist between the JCR:LA-cp rat and other obese strains of animals incorporating either the cp gene or the fa gene, only the JCR:LA-cp males develop both atherosclerosis and ischemic myocardial lesions.[7-9,58] Important metabolic differences between the JCR:LA-cp rat and the fatty Zucker do exist. Obese JCR:LA-cp animals show an impaired glucose tolerance which is greater than that observed in the fatty Zuckers.[58] The hyperinsulinemia observed in the cp/cp male is more severe than in the male or female fatty Zucker rats which are more like the cp/cp females. Although both strains of rats are hyperlipidemic, the obese JCR:LA-cp rats are more severely affected.[58]

Metabolic Characteristics of Rats with Lesions of the Ventromedial Hypothalamus

Ventromedial hypothalamic- (VMH) lesioned animals become obese, hyperphagic, hyperinsulinemic, and insulin resistant.[63,64] VMH lesions do not result in obesity in rats previously treated with streptozotocin which suggests the existence of a neurally mediated VMH influence on the pancreatic β cells.[64] It has also been demonstrated that acute hyperinsulinemia and obesity resulting from bilateral lesions of the VMH can be reversed by vagotomy.[64] It has been hypothesized that the hyperinsulinemia observed in the fatty-Zucker rat is similarly mediated by the vagus nerve. However, vagotomy fails to reverse obesity and hyperinsulinemia in these rats.[58]

Metabolic Characteristics of the db/db Mouse:

The diabetes (db) mutation is located on chromosome 4 and in the homozygote (db/db) results in hyperphagia, obesity, insulin resistance, hyperglycemia, and a syndrome which is similar to NIDDM in man.[52] Genetic analysis has shown that multiple genes are responsible for the expression of disease in this strain of mouse.[52] These mice have proved to be useful models for the study of the molecular basis of insulin resistance and diabetes.[52]

SUMMARY

Understanding the study of the insulin resistance syndrome requires a knowledge of a wide range of disciplines ranging from physiology to molecular biology. The origins of the syndrome remain to be elucidated. However, the growing recognition of insulin resistance as a major cause of human disease coupled with the availability of animal models may serve to promote more research in this area. The aims of this study were to identify the primary site of insulin resistance in the JCR:LA-cp rat and to examine the insulin receptor gene for gross changes which could possibly result in an alteration of receptor function.

CHAPTER 3

MATERIALS AND METHODS

ANIMALS

Rats of the JCR:LA-cp strain were bred in our established colony at the University of Alberta as previously described.[8,9] The rats were housed in polycarbonate cages on wood chip bedding at 20°C and 50-55% relative humidity. Lighting was on from 0600 to 1800 hrs each day. Food was available freely at all times and was Wayne Rodent Blox (Harlan Sprague Dawley Inc., Madison, WI). This is a grain based diet which has been determined to have a total lipid content of approximately 2.5% by weight.[65] Only three homozygous genotypes, +/+ (lean) males, cp/cp (obese) males, and cp/cp females, were studied as +/+ females have been found to be similar in metabolic response to +/+ male rats. All care and treatment of the animals was in accordance with the Guidelines of the Canadian Council on Animal Care and the subject of prior review and approval by the Health Sciences Animal Welfare Committee.

PROCEDURE

All metabolic studies were performed in a post-absorptive state between 1000 and 1400 hrs during the light phase of the animals. The animals were anesthetized with halothane in oxygen and maintained under anesthesia using a mask on a heated table. Cannulae (PE 50, Becton Dickinson and Company, Parsippany, NJ) were inserted into the femoral

artery or vein, as appropriate. The portal vein was exposed through a midline laparotomy.

2-Deoxy-D-Glucose Clearance Studies

Rats were given a 10 μCi bolus injection (0.5 mL of a 20 μCi/ml stock solution) of tracer levels of 2-[^{3}H]-deoxy-D-glucose (Du Pont Canada Inc., Mississauga, Ont.) into the portal vein. Blood was sampled at 2, 5, 10, 20, 30, 40, 50, and 60 minutes via a femoral artery cannula.

Glucose Uptake at Basal Insulin Levels

Rats were given a 10 μCi bolus injection (0.5 mL of a 20 μCi/ml stock solution) of 2DG into either the portal vein or the jugular vein. A cardiac puncture was performed after ten minutes and the animal exsanguinated into a syringe. Samples of liver, diaphragm, heart, brain, epididymal fat, perirenal fat, and subcutaneous fat were taken from each rat and maintained on ice. Following dissection, 200 mg of each tissue was weighed and solubilized in 1 ml of Solvable (Du Pont Canada Inc., Mississauga, Ont.) The samples were heated at 60°C until solubilization was completed, then 15 ml of Atomlight (Du Pont Canada, Inc. Mississauga, Ont.) was added to the solubilized tissue and allowed to stand 48 hrs before counting. Following initial counting, internal standard (^3H_2O, $5.6x10^4$ dpm in 30 μl, Du Pont Canada Inc., Mississauga, Ont.) was added to the vials and allowed to stand another 48 hrs before final counting. The results, expressed

as dpm/g of tissue, were normalized to the brain and are expressed as relative uptake (i.e. brain = 1.0).

Glucose Uptake under Hyperinsulinemic Conditions

Blood was sampled from the arterial cannula through a Luer stopcock directly into a 100 µl heparinized capillary tube. A 1 ml sample was taken for determination of initial plasma glucose and insulin concentrations. Infusion of insulin (Insulin-Toronto, Connaught Novo, Willowdale, Ont., in normal saline) was started at 22 µl/min (2 U/ml insulin) with an initial priming bolus of 77 µl/min over a 3-minute period. Infusion of unlabelled glucose (9% w/v solution) was initiated after 3 minutes at a rate estimated to maintain the initial plasma glucose concentration. Blood was sampled every 5 minutes for determination of plasma glucose concentration. The glucose infusion rate was adjusted as appropriate following each determination until a steady state plasma glucose concentration within 10 mg/dl of the initial value had been reached. Once euglycemia was achieved, (approximately 30 minutes) a bolus injection of tracer levels of ^{3}H-2DG (10 µCi in 0.5 ml) was injected into the portal vein. After a 10-minute period final blood samples were taken for plasma insulin and radioactivity levels. Exsanguination was performed through cardiac puncture, and tissues were dissected, processed, and counted as described above.

Analytical Methods

Plasma glucose was measured using a rapid glucose oxidase technique (Beckman Instruments, Brea, CA). Insulin was measured by a double antibody radioimmunoassay[66] at the Muttart Diabetes Research and Training Centre Core Laboratory using Pharmacia reagents and rat insulin standards. All samples were counted in a Beckman LS100 liquid scintillation counter, with tissue samples processed as described above. Plasma samples were counted using Ready Cap solid scintillation medium (Beckman Instruments, Fullerton, CA).

Statistical Analyses

Curve fitting was by nonlinear technique using SigmaPlot[67] and the two-compartment rate equation, $[2DG] = ae^{-k1t} + be^{-k2t}$. The results were analyzed by testing of group means by paired and unpaired *t*-test using Systat.[68] A value of $p<0.05$ (two-tailed) was considered to be significant.

Insulin Receptor DNA Studies

Animals were JCR:LA-corpulent rats and included +/+, +/cp, and cp/cp female rats (n=1, each genotype). Sprague Dawley female rats were used as rat controls (n=2). Heart punctures were performed under halothane anesthesia, and the animals were exsanguinated into a 10 ml syringe containing Na_2EDTA (1 mg/ml blood). A human blood sample was also used

as a control. DNA was extracted from each sample using the method of Madisen et al.[69] White blood cell lysates were prepared from each sample using a Tris/ammonium chloride mixture (0.155 M NH_4Cl:0.17 M Tris, pH 7.65, 9:1) and a lysis buffer containing 0.2% SDS, 1 M NaCl, 40 mM EDTA, and 100 mM Tris, pH 8.0. DNA was extracted from the lysates two times using neutralized phenol. The aqueous phase was extracted once more with chloroform:isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase by the addition of 0.1 volumes of 4 M ammonium acetate followed by 2.5 volumes of 95% ethanol at room temperature. The DNA was removed to a clean tube and allowed to air dry for 30 minutes. DNA was redissolved in low TE (10 mM Tris, 1 mM EDTA) in the cold on a rocker for 2 days. The DNA concentration was measured in a spectrophotometer at 260 nm. Restriction digests consisted of 20 μg DNA, medium restriction buffer,[70] 4 mM spermidine, and 80 U EcoRI. Digests were incubated overnight at 37°C. DNA was precipitated overnight with 4 M ammonium acetate and two volumes of cold absolute ethanol. Genomic DNA was separated electrophoretically along with λ-HindIII on a 1% agarose gel consisting of agarose (Schwarz/Mann Biotech, Cleveland, OH) and TAE.[70] The gel was stained by including ethidium bromide (0.5 μg/ml) in both the gel and the running buffer and photographed. After depurination in 0.25 M HCl, the DNA was transferred to Genescreen Plus (Du Pont Canada Inc., Mississauga, Ont.) in 0.4 M NaOH.[71] The membrane was neutralized in 1 M NaCl, 1 M Tris, pH 7.0, prehybridized in 6 x SSC, 10% dextran sulfate, 1% SDS, 5 mg/ml denatured salmon sperm DNA, and hybridized by the addition of approximately 10^6 cpm/ml of a ^{32}P-dCTP-oligolabeled[72] human insulin receptor cDNA[19] at 65°C. The blot was washed in 0.5 x SSC, 0.1% SDS at

65°C. The bands were visualized by autoradiography for 48 hrs on Kodak X-OMAT film with an intensifying screen.

CHAPTER 4

RESULTS

Table 1 summarizes the fasting plasma glucose and insulin levels in the three genotypes, male +/+, and male and female cp/cp at both 3 and 6 months of age. These values are similar to those reported by us in previous studies. They show fasting normoglycemia in all genotypes, but elevated insulin levels in the cp/cp rats that are more marked in the males. Final insulin values for experiments done under hyperinsulinemic conditions show that supramaximal insulin levels were achieved during the experiment. Final insulin levels for the three genotypes were not statistically different from one another.

Figure 1 illustrates the clearance of 2DG in 3-month-old rats of the three genotypes. Clearance was exponential and biphasic and the lines in Fig 1 have been fitted to the data assuming two first-order decays. The rate constants are shown in Table 2. In all cases the smaller rate constant (k_2) was approximately one-tenth that of the larger (k_1). In the case of both rate constants, the +/+ male rats showed greater values than the cp/cp rats, but the apparent differences between genotypes were not significant ($p>0.05$). There were no statistically significant differences in the clearance of 2DG between genotypes under the basal insulin conditions. The 10-minute period used for the tissue uptake studies covered the initial period during which approximately 50% of the 2DG was taken up from the plasma.

The relative uptake of 2DG in various organs following portal vein injection in 3-month-old rats under basal insulin conditions is shown in Figure 2. Raw brain uptake values (mean±standard deviation) in dpm/mg *1000 for

+/+males, cp/cp males, and cp/cp females respectively, were 221.9±52.9, 183.0±26.0, and 239.2±30.0. The male and female cp/cp animals showed no significant differences in glucose uptake in the liver, diaphragm, heart or epididymal fat compared to the lean males under these conditions. Under basal conditions glucose uptake in the perirenal fat was decreased in the cp/cp males ($p<0.001$) in comparison to the +/+ male animals. The subcutaneous fat depot in both corpulent genotypes shows far less uptake ($p<0.001$) than the same fat depot in the +/+ animals. The raw data, expressed as dpm/g, exhibited the same patterns of results, but with somewhat greater animal-to-animal variance.

Figure 3 shows 2DG uptake in various organs following portal vein injection in 3-month-old rats under hyperinsulinemic conditions. Raw brain uptake values (mean±standard deviation) in dpm/mg *1000 for +/+males, cp/cp males, and cp/cp females respectively, were 140.5±35.4, 152.8±12.7, and 266.4±40.1. In the +/+ rats the elevated insulin levels resulted in relative changes in glucose uptake, but no change in the rate of uptake in the brain (data are not shown). Increased levels of insulin had no effect on relative uptake in the liver, heart, or fat depots. However, there was a large increase in uptake in the muscle ($p<0.001$). Similarly, the cp/cp rats, both male and female showed no significant change in uptake in the liver. The cp/cp females showed an increase in muscle uptake ($p<0.001$) that was significantly less than that shown by the +/+ rats, as well as an increased uptake in the heart ($p<0.05$). Decreased uptake in the epididymal ($p<0.01$) and the subcutaneous ($p<0.05$) fat depots was also observed in the corpulent females. The cp/cp males, in contrast, showed no response to increased levels of insulin in any organ.

The uptake of 2DG in various organs following portal vein injection in 6-month-old animals under basal insulin conditions is shown in Figure 4. The subcutaneous fat of the +/+ male rats did not show the greater uptake seen in the 3-month-old animals. The male and female cp/cp rats show lower uptake in the subcutaneous depot. However, at this age the differences compared to the +/+ rats are not statistically significant. The uptake by perirenal fat only in the cp/cp females was low in comparison to the +/+ animals ($p<0.01$). Glucose uptake in the liver of the cp/cp male rats was increased in comparison to the +/+ males ($p<0.05$). Statistical comparisons between 3- and 6-month-old animals show that glucose uptake in 6-month-old animals is decreased in the perirenal fat ($p<0.001$) and subcutaneous fat ($p<0.05$) in the lean males, and in the epididymal fat ($p<0.05$) and perirenal fat ($p<0.05$) of the female corpulent rats in comparison to the 3-month-old rats. The corpulent males show no decreases in uptake with age, but a small increase in uptake in the liver ($p<0.05$). The 6-month-old +/+ males and cp/cp females also show small increases in uptake in the liver in comparison to the 3-month-old animals, but these differences are not significant.

The relative uptake of 2DG in various organs following jugular vein injection in 3-month-old rats under basal insulin conditions is shown in Figure 5. The male cp/cp rats show no significant differences in glucose uptake in the liver, diaphragm, heart, epididymal fat or perirenal fat compared to the lean males under these conditions. Under basal insulin conditions glucose uptake in the heart ($p<0.001$), epididymal fat ($p<0.05$), and perirenal fat ($p<0.05$) was decreased in the cp/cp females in comparison to the +/+ male animals. The subcutaneous fat depot in both corpulent genotypes shows far less uptake than the same fat depot in the +/+ animals ($p<0.001$). Statistical

comparisons between animals given portal injections versus animals given jugular injections show a decrease in 2DG uptake in the hearts ($p<0.001$) of +/+ male rats given portal injections versus lean males given jugular injections. Corpulent males given portal injections show decreased glucose uptake in the heart ($p<0.05$) and epididymal fat ($p<0.05$) compared to cp/cp males given jugular injections. An increase in glucose uptake in the epididymal fat ($p<0.05$) was observed in cp/cp females given portal injections versus corpulent females given jugular injections.

The uptake of 2DG in various organs following jugular vein injection in 6-month-old animals under basal insulin conditions is shown in Figure 6. The male cp/cp animals show no significant differences in glucose uptake in the liver, diaphragm, or epididymal fat compared to the lean males under these conditions. Glucose uptake in the heart ($p<0.05$), perirenal fat ($p<0.05$) and subcutaneous fat ($p<0.01$) was decreased in the cp/cp males in comparison to the +/+ male animals. The cp/cp females show no significant differences in glucose uptake in the diaphragm, epididymal fat, or perirenal fat under basal insulin conditions. However, increased uptake in the liver ($p<0.05$) and decreased uptake in the heart ($p<0.01$) and subcutaneous fat ($p<0.05$) were observed in the cp/cp female rats compared to the lean males under these conditions. Comparisons between portal versus jugular injections showed an increase in 2DG uptake in the liver with portal injection ($p<0.05$) in both the +/+ male and the cp/cp male rats. Lean males given portal injections showed decreased uptake in the heart ($p<0.01$) and subcutaneous fat ($p<0.05$) in comparison to lean males given jugular injections. Corpulent females given portal injections showed decrease uptake in the perirenal fat ($p<0.05$) and subcutaneous fat ($p<0.05$) compared to cp/cp females given

jugular injections. Statistical comparisons between 3- and 6-month-old animals show that glucose uptake in 6-month-old animals is increased in the subcutaneous fat in the cp/cp females ($p<0.05$). The +/+ and cp/cp male rats show no changes in glucose uptake with age when given jugular injections.

To explore the possibility that the insulin resistance observed in the JCR:LA-corpulent rat is due to a genetic alteration of receptor function and, in particular, gross changes in the insulin receptor gene, we analyzed EcoRI cleaved DNA by Southern blot, using a human cDNA probe for the insulin receptor gene. Southern blot analysis indicates the absence of gross rearrangements, insertions or deletions of the insulin receptor gene (Figure 7). Differences in band intensities observed on the blot are due to differences in the concentrations of DNA loaded into each lane and do not reflect genetic differences between rats. The photograph of the original gel (left) illustrates the differences in DNA concentrations between lanes and is provided for purposes of comparison.

CHAPTER 5

DISCUSSION

Halothane induces a basal state of glucose metabolism in the rat.[73,74] The data on clearance of 2DG, together with the concentrations of glucose and insulin shown in Table 1, are consistent with previous findings and confirm this. The small differences in clearance rates between genotypes are consistent with our previous findings of impaired glucose tolerance in the cp/cp male rat.[15] The results in Table 1 are also consistent with the suggestion that the cp/cp rats are able to largely compensate for the insulin resistance through hyperinsulinemia. The clearance of 2DG from the plasma as shown in Figure 1 has two exponential components. These represent a fast uptake by tissues and a slow clearance from the body via the kidneys.

Under basal insulinemic conditions the cp/cp rats showed essentially identical glucose uptake to the +/+ animals, again confirming that the animals are able to compensate for the insulin resistance. This is also consistent with our findings that the cp/cp rats, both male and female, have very similar rates of glucose turnover under these conditions.[15] The liver of the cp/cp rat is approximately twice as large as that of a +/+ rat, and thus, while the per gram uptakes are the same, the total hepatic uptake in the cp/cp rat is larger than that of the +/+ animal. Correspondingly, the muscle mass of the +/+ rat is larger, and thus, in absolute terms, the cp/cp rat preferentially takes up glucose in the liver and the +/+ rat in the muscle. Despite the greater relative uptake in the perirenal, and especially in the subcutaneous fat of the +/+ rat, the very small size of these depots makes the overall uptake small. Estimates based on the approximately 10-fold greater weight of fat in the cp/cp male

compared to the +/+ male rat, suggest that the total glucose uptake in the fat of the cp/cp males is two to nine times that in the +/+ males. The principal changes in glucose uptake with age (Figure 4) were reductions in the uptake by fat. This most likely reflects the fact that at six months of age all of the rats, and especially the cp/cp animals, show relatively low rates of growth and fat deposition and a much reduced hyperphagia.

Under the influence of high insulin levels, the +/+ rats showed a marked increase in the glucose uptake by muscle. This matches the approximately 100% increase in glucose turnover found previously.[15] Since other tissues showed only small and non-significant increases in glucose uptake, the 3.2-fold increase in muscle uptake would lead to an overall increase in turnover similar to that measured in the earlier study. The cp/cp female rats showed a very significant increase in 2DG uptake in muscle that was smaller than that of the +/+ male rats. This is again consistent with the somewhat smaller insulin-mediated increase in glucose turnover found in the cp/cp females.[15] Under euglycemic insulin clamp conditions the cp/cp male rats showed no insulin-mediated increase in glucose turnover,[15] and the 2DG uptake results (Figure 3) confirm this lack of response. Glucose uptake in tissues other than muscle, at least as inferred from 2DG uptake, is not responsive to increased insulin levels beyond those present under basal conditions. The lack of response to insulin observed in the adipose tissue in both the lean and corpulent genotypes was a very interesting observation and may prove to be very important. Basal insulin levels in cp/cp rats (Table 3) were approximately 30% of the ED_{50} for insulin in these animals, and the levels attained during the euglycemic clamps were approximately 20 times the ED_{50}.[15] It is likely that non-insulin-dependent transporters, such as GLUT1,

are responsible for glucose uptake at the lower basal insulin levels. The present results do not allow for elucidation of the mechanisms involved, although studies of hypoinsulinemic rats and measurement of glucose transporter levels would do so. The results do make it clear that the important peripheral resistance in the cp/cp male rat lies in the muscle.

Glucose uptake studies via jugular vein injection were performed for the sake of completeness and to observe if varying the injection site would lead to major differences in results. No major differences in glucose uptake were observed. Glucose uptake via jugular vein injection under basal insulin conditions was comparable to glucose uptake in the portal vein studies performed under basal conditions. However, glucose uptake in the heart of the cp/cp female rats was significantly increased in comparison to the other genotypes in the jugular vein studies, and it is not obvious why this is the case. It is also not clear why an increase in glucose uptake is observed in the subcutaneous fat of 6-month-old rats in comparison to 3-month-old rats in the jugular studies.The portal vein data were more consistent than data obtained from the jugular vein studies. It is also important to recognize that the portal injection site is closer to the normal physiological status of the animal where exogenous glucose appears first in the portal circulation. The principal difference between the jugular vein studies and the portal vein studies was an increase in glucose uptake by the heart of the three genotypes in the jugular vein studies in comparison to glucose uptake in the portal vein studies. This result was not surprising, as the heart in this case would receive the initial pass of tracer.

The liver plays a central role in the regulation of glucose metabolism. The absence of any insulin mediated change in hepatic glucose uptake

matches the conclusions of DeFronzo.[22] He also suggests that the site of insulin resistance is in the peripheral tissues and primarily the muscle and this is consistent with our finding of muscle insensitivity. However, there is evidence of abnormal hepatic response to insulin in the cp/cp male rat that appears to result in exaggerated diversion of glucose to triacylglycerol synthesis.[75] Further, we have found that insulin binding to membrane receptors in the liver of the cp/cp female rats is 40% lower than in +/+ rats (unpublished results). This suggests that the cp/cp rat may have altered insulin receptor numbers, with resultant effects on hepatic glucose metabolism. Insulin binding in the muscle and adipose tissue of the three genotypes is currently being examined.

The results of our 2DG uptake studies are similar to those reported by Pénicaud et al.[76] in their studies of the fatty Zucker rat. While the fa and cp genes are allelic,[77,78] there are major metabolic differences between fa/fa and cp/cp rats.[79,58] The different strains of obese rats also exhibit very different expressions of overt disease, with the JCR:LA-cp strain being unique in the development of cardiovascular disease.[7,79] The results of Pénicaud et al.[76] were obtained using only female rats, and the study of glucose uptake at 12 weeks of age was confined to muscle and white adipose tissue. Three-month-old fa/fa rats showed markedly reduced glucose uptake in muscle under basal insulin levels compared to Fa/Fa (lean) rats. This result was also demonstrated in a study done by Crettaz et al.[80] which also utilized Zucker females. In contrast, muscle glucose uptake in both corpulent genotypes was comparable to the lean rats under basal insulin conditions. The Pénicaud study showed a very small increase in glucose uptake in adipose tissue in response to high insulin levels in the female Zucker rats which was

not observed in our animals. The lean Zucker rats showed a much greater response to insulin in muscle than the +/+ rats of our strain, while the obese Zucker rats showed a smaller response in muscle than the cp/cp females. The results reported here confirm that the cp/cp male rat of the JCR:LA-cp strain has a greater insulin resistance than the fa/fa rats, and this is consistent with the much greater rates of insulin secretion and levels of hyperinsulinemia observed in the cp/cp male rats.[58] This extreme disturbance of insulin and glucose metabolism appears to be a critical element in the spontaneous development of cardiovascular disease in this strain.[79]

Southern blot analysis of the insulin receptor gene in our rats demonstrates that insulin resistance in the JCR:LA-cp rats is not associated with large rearrangements, insertions, or deletions in the insulin receptor gene. This suggests that although insulin receptor mutations might exist in these rats, they would be small deletions or insertions or point mutations which could not be detected by this type of analysis. A reduction in insulin receptor gene transcription rate or a reduced stability of insulin receptor mRNA could also account for our observations. Clearly a more detailed analysis of the insulin receptor gene, mRNA transcripts and perhaps the glucose transporters is required before any more conclusions about the origin of the insulin resistance in the JCR:LA-cp rat can be made.

Table 1: Insulin and Glucose Concentrations in the Plasma of JCR:LA–cp Rats

Group	Insulin (mU/L)	Glucose (mg/dl)	Body Weight (g)
3-month-old, basal insulin condition:			
+/+ male	40 ± 19	135 ± 22	307 ± 33
cp/cp male	314 ± 121	150 ± 23	456 ± 54
cp/cp female	236 ± 76	175 ± 33	358 ± 20
6-month-old, basal insulin condition:			
+/+ male	24 ± 0.2	136 ± 12	374 ± 18
cp/cp male	350 ± 47	137 ± 18	658 ± 44
cp/cp female	227 ± 101	189 ± 23	555 ± 31
3-month-old, hyper-insulinemic condition:			
+/+ male	59000 ± 26000		
cp/cp male	34000 ± 12000		
cp/cp female	74000 ± 29000		

Values are mean ± SD. There were 5 rats in each group.

Table 2. First Order Rate Constants for the Clearance of 2-deoxy-D-glucose from the Plasma of JCR:LA-cp Rats

Genotype	$k_1 \cdot min^{-1}$	$k_2 \cdot min^{-1}$
+/+ male	0.180 ± 0.016	0.015 ± 0.008
cp/cp male	0.161 ± 0.088	0.0053 ± 0.0164
cp/cp female	0.120 ± 0.071	0.0107 ± 0.0187

Values are mean ± SD. There were 5 rats in each group.

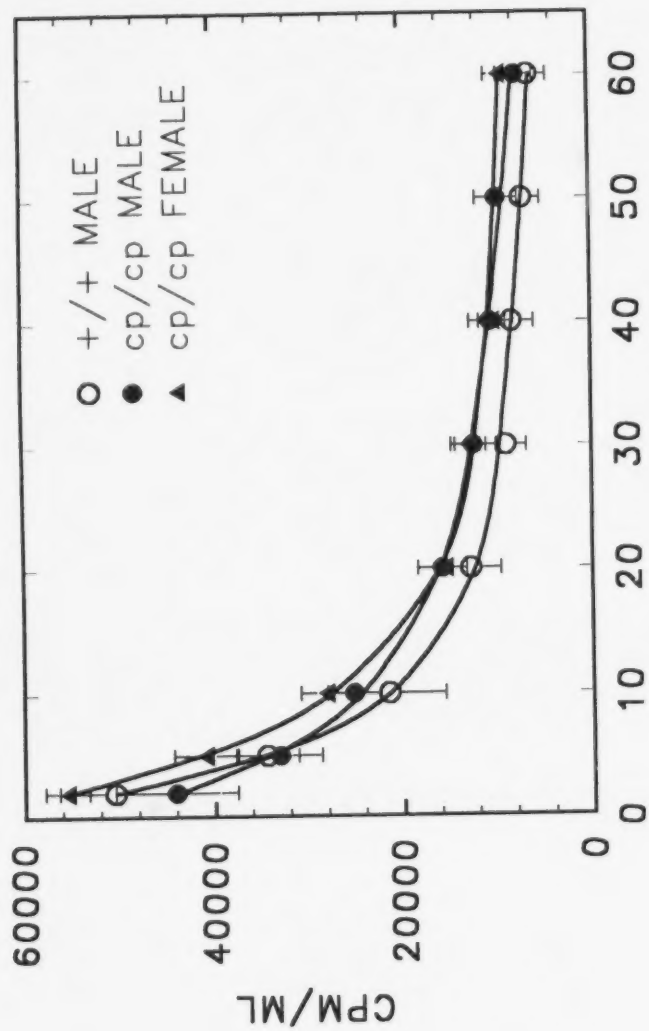


Figure 1. 2-[^{3}H]-deoxy-D-glucose clearance in 3-month-old rats. Data are means ± S.D. for 5 animals at 3 months of age. Lines were calculated as described in the text.

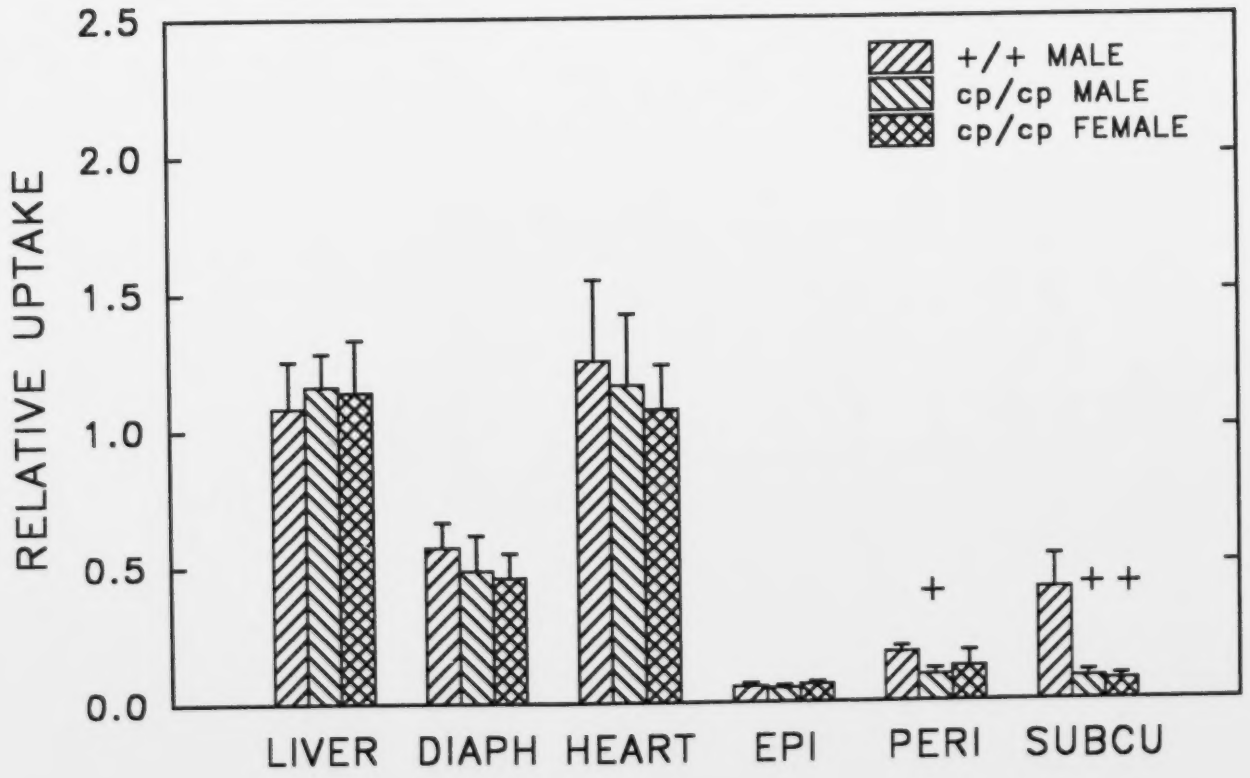


Figure 2. 2-[^{3}H]-deoxy-D-glucose uptake via portal vein injection in 3-month-old rats under basal insulin conditions. Data are normalized rat by rat against brain uptake. Each value represents the mean ± S.D. for 6 animals at 3 months of age. + $p<0.001$ *vs.* +/+ males. DIAPH, diaphragm; EPI, epididymal fat; PERI, perirenal fat; SUBCU, subcutaneous fat.

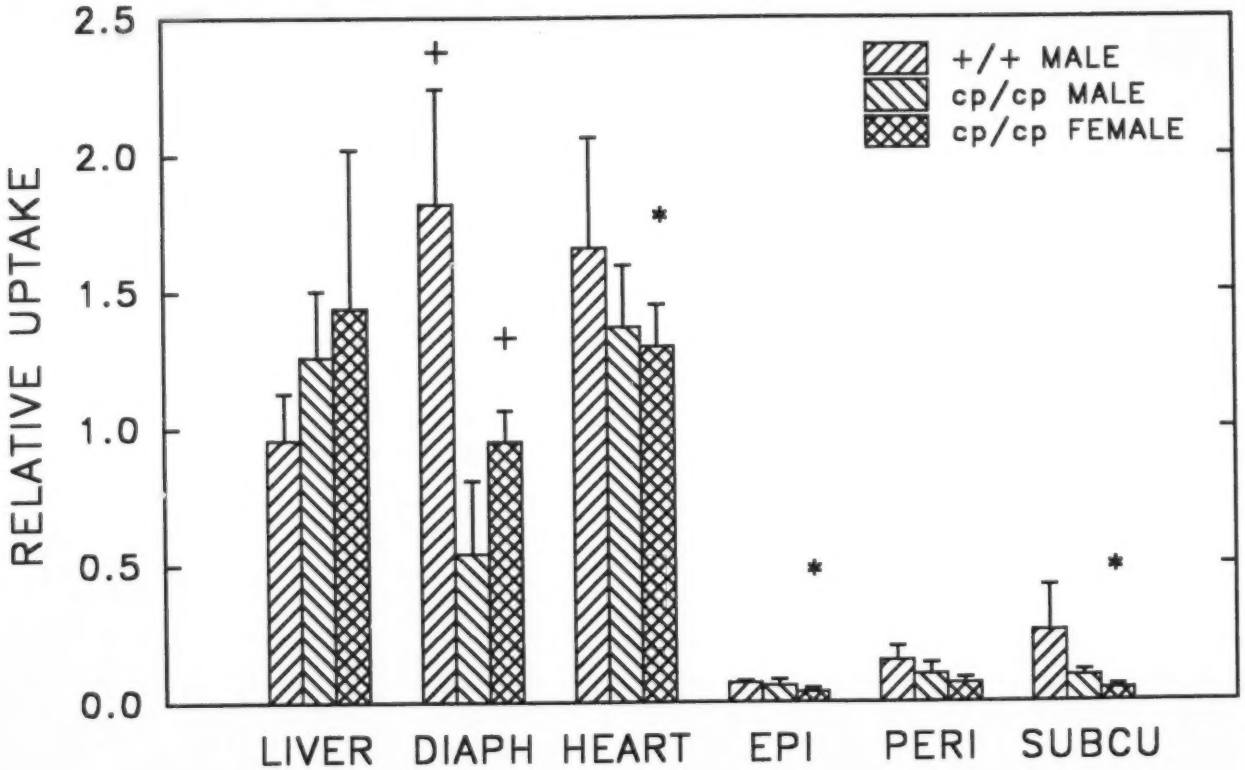


Figure 3. 2-[^{3}H]-deoxy-D-glucose uptake via portal vein injection in 3-month-old rats under hyperinsulinemic conditions. Data are normalized rat by rat against brain uptake. Each value represents the mean ± S.D. for 5 animals at 3 months of age. * $p<0.05$, + $p<0.001$ *vs.* 3-month-old rats under basal insulin condtions. Tissue identification is as in Figure 2.

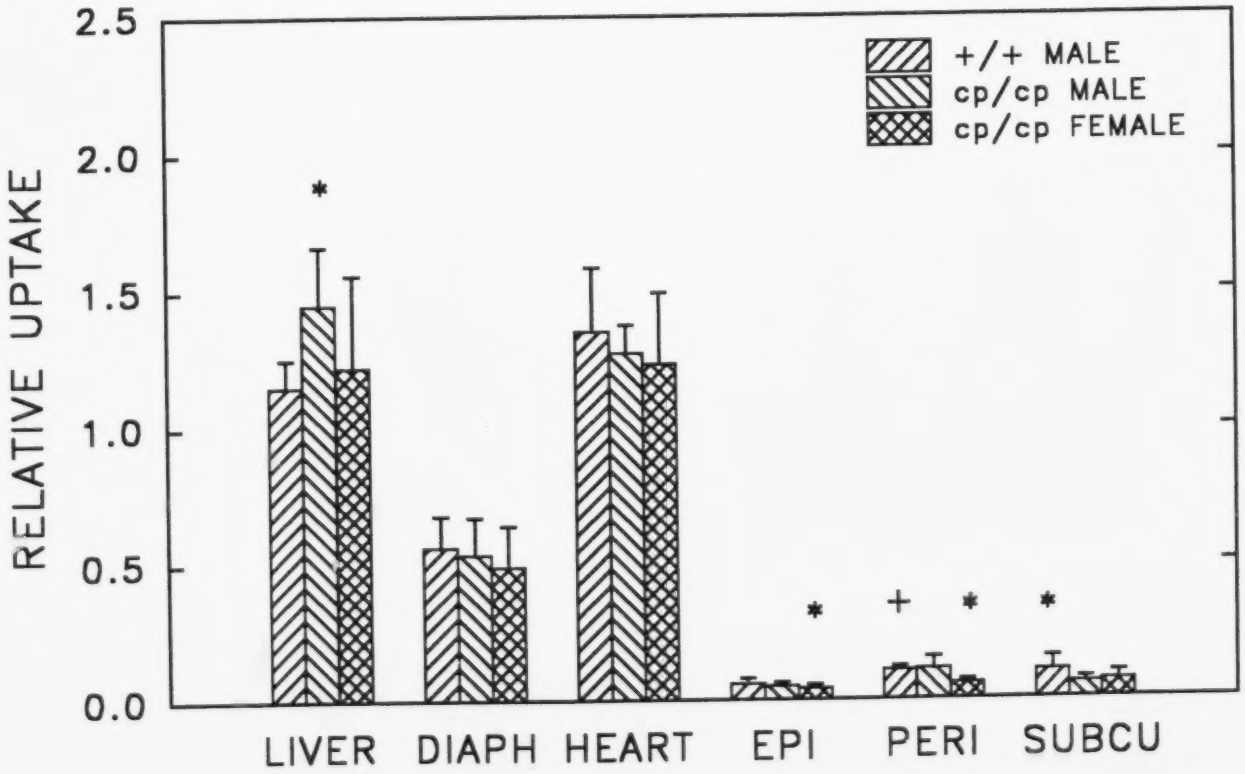


Figure 4. 2-[^{3}H]-deoxy-D-glucose uptake via portal vein injection in 6-month-old rats under basal insulin conditions. Data are normalized rat by rat against brain uptake. Each value represents the mean ± S.D. for 6 animals at 6 months of age. * $p<0.05$, + $p<0.001$ *vs.* 3-month-old rats under basal insulin conditions.

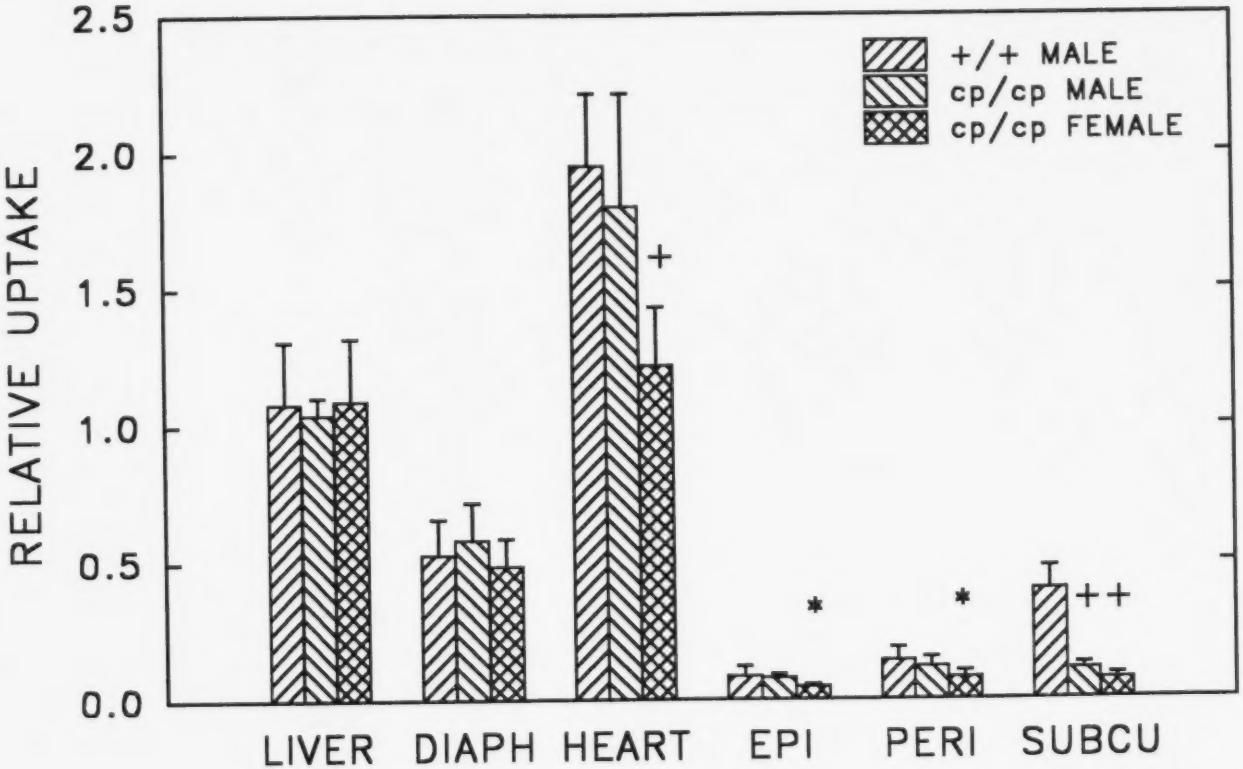


Figure 5. 2-[^{3}H]-deoxy-D-glucose uptake via jugular vein injection in 3-month-old rats under basal insulin conditions. Data are normalized rat by rat against brain uptake. Each value represents the mean ± S.D. for 6 animals at 3 months of age. * $p<0.05$, + $p<0.001$ *vs.* +/+ males.

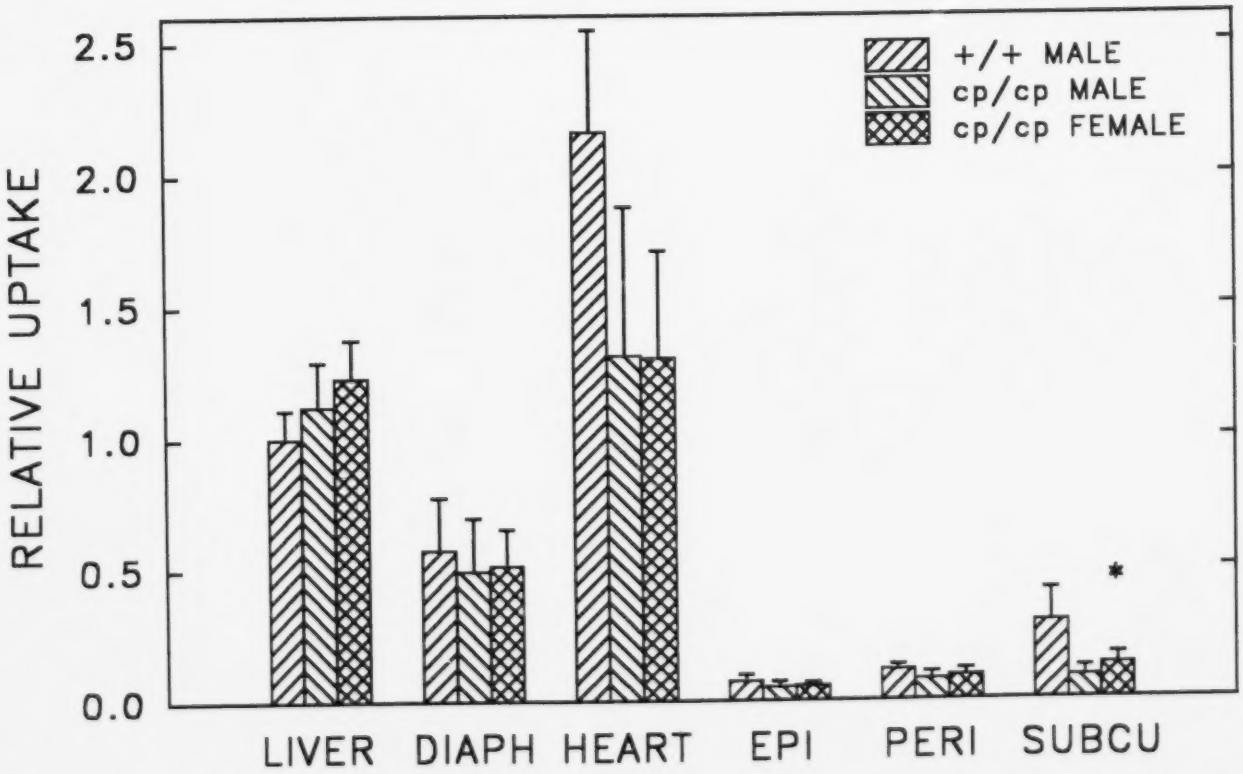
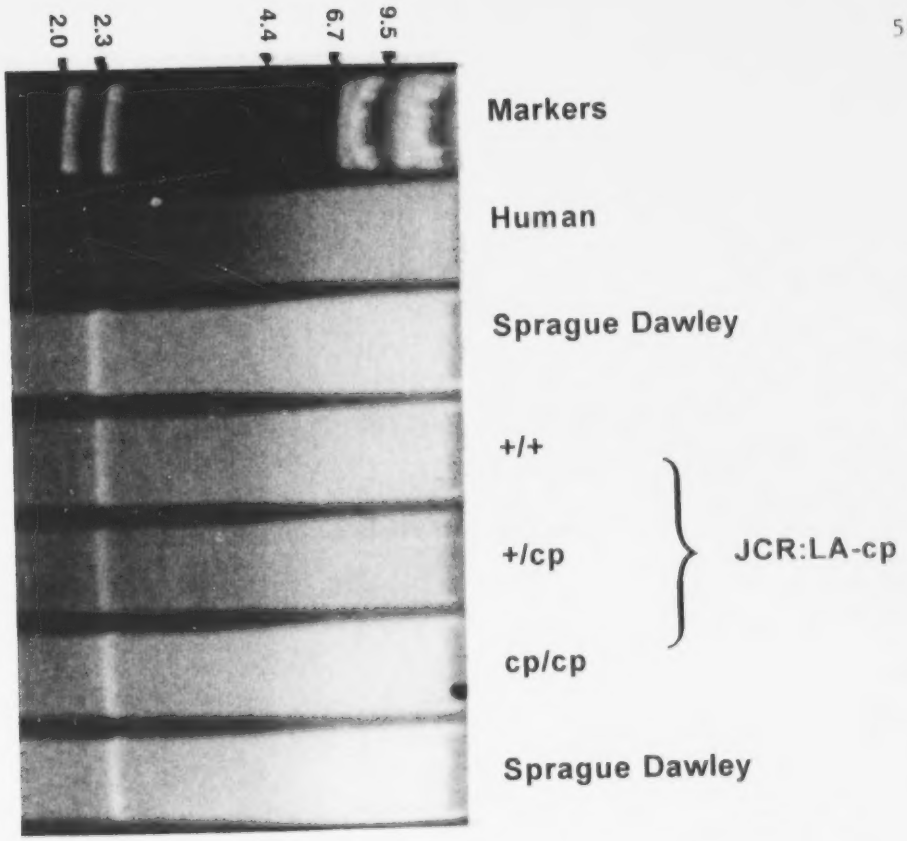
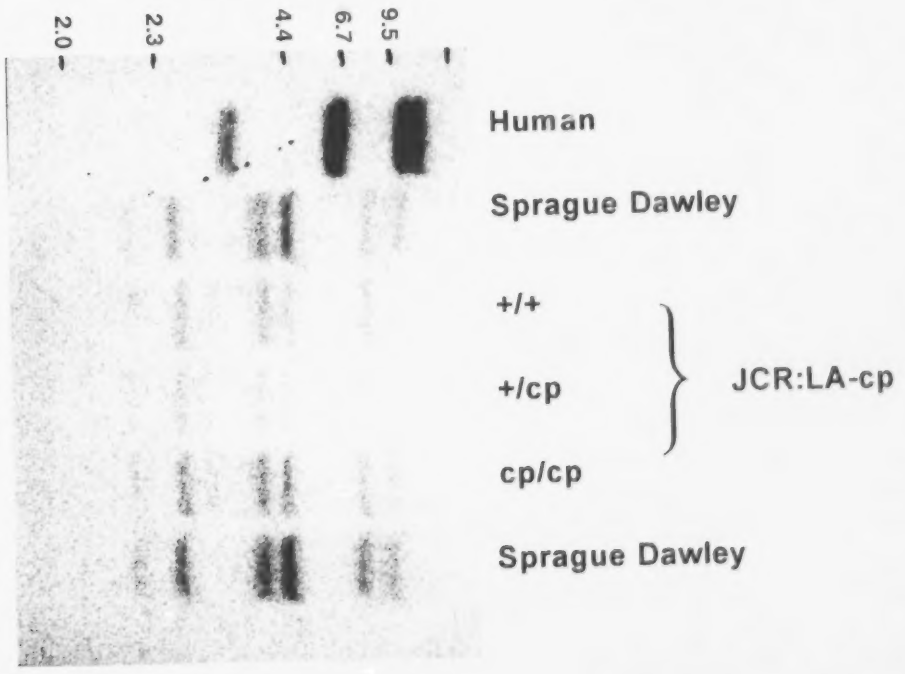


Figure 6. 2-[^{3}H]-deoxy-D-glucose uptake via jugular vein injection in 6-month-old rats under basal insulin conditions. Data are normalized rat by rat against brain uptake. Each value represents the mean ± S.D. for 6 animals at 6 months of age. * $p<0.05$ *vs.* 3-month-old rats under basal insulin conditions.

Figure 7. **Southern blot analysis of restriction endonuclease–digested DNA from JCR:LA–cp rats and Sprague Dawley controls.** Molecular weight markers (in kilobases) are indicated on the left. The figure shows the original photograph of the gel (top) and an autoradiogram (bottom) exposed 45 hrs at -70°C. Further details are given in the Methods section.


2.0
2.3
4.4
6.7
9.5
Markers
Human
Sprague Dawley
+/+
+/cp
cp/cp
JCR:LA-cp
Sprague Dawley


2.0
2.3
4.4
6.7
9.5
Human
Sprague Dawley
+/+
+/cp
cp/cp
JCR:LA-cp
Sprague Dawley

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